oil obtained amounted to 16% of the weight of the lignin used. Fifty-five per cent. of the oil was alkali-soluble, and in this fraction guaiacol was definitely identified. A phenol containing the methoxyl group and yielding a 3,5-dinitrobenzoyl derivative melting at 110° was also obtained but was not further identified.

2. The isolation of catechol and guaiacol as degradation products of lignin is believed to lend support to the hypothesis that lignin contains an aromatic nucleus.

3. The evolution of carbon dioxide in the zinc dust distillation of lignin in an atmosphere of hydrogen indicates that, in all probability, the lignin molecule contains at least one carbon atom directly united to two oxygen atoms. This may indicate the presence of either a carboxyl group, free or esterified, or a lactone group in the lignin molecule.

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[185TH CONTRIBUTION FROM THE COLOR AND FARM WASTE DIVISION, BUREAU OF CHEMISTRY AND SOILS, U. S. DEPARTMENT OF AGRICULTURE]

THE PRODUCTION OF KOJIC ACID BY ASPERGILLUS FLAVUS

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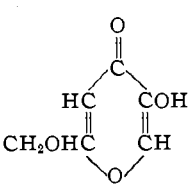
In the course of a survey of the action of a number of fungi on solutions of xylose and dextrose it was observed that the culture liquors from a strain of *A. flavus* on both sugars gave a deep red coloration upon the addition of ferric chloride solution. Continuous extraction of these cultures with ether yielded a quantity of material crystallizing as colorless needles, soluble in water, methanol, ethanol and ethyl acetate. It was identified as kojic acid by its melting point (152.6° (corr.); Yabuta, 152°; Mauer, 152°; Kinoshita, 154°), by the melting point of the monobromo derivative (161° (corr.); Yabuta, 159–160°), and by analysis of the insoluble copper salt (found, average of six determinations, 22.40% CuO. Calcd. for $Cu(C_6H_5O_4)_2 \cdot {}^1/_2H_2O$, 22.44%; Mauer found 22.12% CuO). The crystalline products obtained from the xylose and dextrose solutions were identical in all their properties.



Kojic acid was first isolated by Saito[1] from mycelia of *A. oryzae* which had been cultured on steamed rice. He thought it to be identical with β-resorcylcarboxylic acid. Shortly thereafter Yabuta[2] undertook an extensive investigation of the substance, gave it the name kojic acid and.

[1] K. Saito, *Botan. Mag.* (Japan), **21**, 249 (1907).

[2] T. Yabuta, *J. Coll. Agr., Tokyo*, **5**, 51 (1912); *8th Int. Cong. Applied Chem., Appendix*, **25**, 455 (1912); *J. Chem. Soc. Japan*, **37**, 1185 (1916) (in Japanese); see *Chem. Abs.*, **17**, 1475 (1923); *J. Chem. Soc.*, **125**, 575 (1924).

finally, in 1924, definitely established its constitution. Traetta-Mosca reported the formation of the same compound by *A. glaucus* from sucrose, levulose, dextrose and glycerol and also noted that it was fermented to ethanol by yeast.[3] Wijkman, using an unnamed strain of Aspergillus and employing quartz or paraffined glass vessels as culture flasks, consistently obtained the acid from sucrose.[4] Kinoshita has observed that a deficiency in the supply of nutrient nitrogen resulting from the use of cobalt ammines as nitrogen sources caused an increased production of kojic acid by a strain of *A. oryzae*. Thus, when cobalt purpureo chloride in a concentration of 0.5% was used as the source of nutrient nitrogen, 33% yields by weight were obtained from 10% sucrose solutions in twenty-five days.[5] Tamiya found that a P_H of 5.5 was most favorable to the formation of the acid by *A. oryzae* and later reported the production of the compound from sucrose by the following Aspergilli: *oryzae*, *flavus var.*, *gymnosardae*, *awamori*, *candidus*, *clavatus*, *fumigatus* and *giganteus*.[6] Challenger, Klein and Walker pointed out that, from the standpoint of molecular structure, since dextrose yields kojic acid, arabinose might by analogy be expected to give rise to pyromeconic acid. To test this hypothesis, they cultured a strain of *A. oryzae* on solutions of highly purified arabinose and xylose. Kojic acid was the only pyrone compound found in the culture liquors. Moreover, they recovered it from the pentose cultures in yields of the same order of magnitude as those obtained from dextrose.[7] Katagiri and Kitahara found the optimum conditions for the formation of kojic acid by *A. oryzae* to be in 5% dextrose solutions containing 0.05% ammonium sulfate, at P_H 2.4.[8] Corbellini and Gregorini made a study of the formation of the acid from various carbon sources by different strains of *A. flavus* and concluded that the pyrone nucleus was synthesized from substances containing a chain of three carbon atoms resulting from the fragmentation of larger molecules such as the pentoses and hexoses.[9] Starting with acetobromoglucose, Mauer has recently succeeded in synthesizing kojic acid, confirming the structure established for the natural product by Yabuta.[10]

Continuous extraction with ether of a ten-day culture of *A. flavus* No. 3538 on 15% solutions of commercial dextrose yielded 22.69 g. of pure kojic acid per 100 g. of pure dextrose originally present in the culture solution. The comparatively high yield obtained in this short period of culture led

[3] F. Traetta-Mosca, *Ann. chim. appl.*, **1**, 4777 (1914); F. Traetta-Mosca and M. Preti, *Gazz. chim. ital.*, **51**, 269 (1921).

[4] N. Wijkman, *Z. physiol. Chem.*, **132**, 104 (1924).

[5] K. Kinoshita, *Acta Phytochimica*, **3**, 31 (1927).

[6] H. Tamiya, *ibid.*, **3**, 51 (1927). H. Tamiya and T. Hida, *ibid.*, **4**, 343 (1929).

[7] F. Challenger, L. Klein and T. K. Walker, *J. Chem. Soc.*, 1498 (1929).

[8] H. Katagiri and K. Kitahara, *Bull. Agr. Chem. Soc. Japan*, **5**, 38 (1929).

[9] A. Corbellini and B. Gregorini, *Gazz. chim. ital.*, **60**, 244 (1930).

[10] K. Mauer, *Ber.*, **63**, 25 (1930).

to an investigation of some of the more important variables affecting the fermentation, such as nutrient salts, temperature and concentration of dextrose, with a view to increasing the yields to a point which would make the process of industrial interest and stimulate a search for uses for this hitherto unavailable pyrone.

Experimental

The organism (No. 3538) was secured from the collection of Dr. Charles Thom, where it had been kept in continuous culture since 1914. Its appearance was typical of the *flavus–oryzae* group and its mycological characteristics are to be described in a forthcoming publication.

For the most part, the experiments were carried out in 200-cc. pyrex glass Erlenmeyer flasks as culture flasks. The dextrose was of commercial grade and contained approximately 91.5% dextrose, 8.0% water, 0.5% dextrin and other non-reducing carbohydrates. Nutrient salts were added in the following concentrations, except when stated otherwise.

	G./liter of dextrose solution
$MgSO_4 \cdot 7H_2O$	0.500
KCl	.100
H_3PO_4	.054
NH_4NO_3	1.125

Seventy-five cc. of culture solution per flask was used to give a surface area/volume ratio of approximately 0.4. The solutions were sterilized for fifteen minutes at 15 lb. steam pressure and after cooling were inoculated with spores of the organism. All cultures were run in duplicate or triplicate.

At the conclusion of the fermentation the contents of the culture flask were heated and filtered with suction through a weighed filter paper. The mycelium was thoroughly washed with three 25-cc. portions of hot distilled water, the washings were added to the culture liquors and the volume of the combined solutions was noted. The filter paper and mycelium were placed in a tarred weighing bottle, dried at 90° for two days and the weight of the dry mat was obtained. Reducing sugar, calculated as dextrose, was determined by the Shaffer–Hartman method, using the citrate–carbonate reagent. In the presence of kojic acid it was impossible to determine the true end-point in the thiosulfate titration when starch was used as the indicator because of the difficulty in discharging the starch–iodine color. For this reason starch was not used, and the typical sharp color change from green to blue was utilized to indicate the disappearance of the iodine. However, in experiments where the concentration of kojic acid exceeded 3 g. per 100 cc. of solution, control titrations indicated that a true end-point was not obtained. In such cases it was necessary to dilute the sample so that the above-mentioned concentration of acid was not

exceeded or, where the concentration of sugar was low, to remove the acid as the insoluble copper salt and make the determination on the filtrate. Since kojic acid reduces hot alkaline copper solution, a correction, determined by the quantity of acid present, was applied to the values obtained for reducing sugars. The reduction values of kojic acid in terms of mg. of copper were determined and are given in Table I.

TABLE I

KOJIC ACID REDUCTION VALUES

Kojic acid, mg	5	10	20	30
Cu, mg	9.5,	17.0,	35.0,	52.0

The acid was determined by neutralization of an aliquot of the culture liquors with dilute alkali and precipitation of the partially hydrated copper salt with $N/10$ cupric acetate. The precipitate was dried at 100°, weighed and the acid calculated on the basis of $Cu(C_6H_5O_4)_2 \cdot {}^1/_2H_2O$. In the absence of other acids, kojic acid can be estimated accurately by titration with dilute alkali, with alizarin orange R as the indicator. However, in our experiments the culture solutions were invariably colored a deep yellow, which interfered somewhat with the observation of the color change of the indicator.

The yields were calculated as the ratio of the dextrose required to give the quantity of kojic acid formed to the total dextrose originally present in the culture solution.

At the outset of these experiments it became apparent that the source of nutrient nitrogen was of prime importance among the factors governing the formation of kojic acid. The effect on acid production of some nitrogen-containing salts is shown in Table II. Ammonium sulfate was unsatisfactory, and it is of interest that its use almost completely suppressed sporulation. Ammonium nitrate was most satisfactory. The effect of variation in its concentration is given in Table III. Nitrates, while supporting a vigorous vegetative development, were found to have an adverse effect on acid formation when used in conjunction with the standard salt

TABLE II

EFFECT OF SOURCE OF NUTRIENT NITROGEN

Temperature, 22°; duration of culture, 14 days; concentration of commercial dextrose, 15%; volume, 75 cc.

N source	G. N/liter	Yield of kojic acid G.	Yield of kojic acid %	Mycelium, g.
$(NH_4)_2SO_4$	0.70	0.90	10.0	0.320
$(NH_4)_2SO_4$	1.75	1.04	11.7	.342
$(NH_4)_2HPO_4$	0.70	1.45	16.3	.526
$(NH_4)_2HPO_4$	1.75	1.65	18.6	.558
NH_4NO_3	0.70	1.66	18.6	.432
NH_4NO_3	1.75	1.45	16.3	.461
$NaNO_3$	0.70	0.08	1.0	.277

TABLE III

EFFECT OF VARIATION IN CONCENTRATION OF AMMONIUM NITRATE

Temperature, 25°; duration of culture, 13 days; concentration of commercial dextrose, 15%; volume, 75 cc.

NH_4NO_3, g./liter	Yield of kojic acid G.	%	Sugar consumed, g.	Yield of kojic acid, based on sugar consumed, %	Mycelium, g.
0.142	0.430	4.8	1.4	39.0	0.124
.281	2.48	27.9	5.5	57.2	.146
.563	2.69	30.2	6.4	53.2	.204
.750	2.65	29.8	7.2	46.7	.235
1.125	2.61	29.4	6.7	49.4	.282
2.250	2.40	27.0	6.1	49.9	.261
4.500	2.03	22.8	Undetermined	..	.245

solution, as noted in Table II, but when type and concentration of the added salts were changed, a decided increase in the quantity of kojic acid produced was noted, as will be observed in Table IV. That this increase was not due to the direct nutritive value or to physical effects of these added salts is evident from the results with ammonium nitrate, where a slightly lower yield was obtained than in experiments where the standard salt solution was used. With both nitrates and ammonium salts, however, in all cases the use of the B solution induced an appreciably greater vegetative development.

Temperature was found to have a decided effect on the quantity of kojic

TABLE IV

COMPARISON OF AMMONIUM NITRATE AND SODIUM NITRATE USED WITH TWO NUTRIENT SALT SOLUTIONS

Temperature, 30°; duration of culture, 12 days; commercial dextrose, 20%; culture vol., 75 cc.

A—Salts (KCl 0.1, H_3PO_4 0.054, $MgSO_4 \cdot 7H_2O$ 0.5 g./liter.)
B—Salts (KH_2PO_4 1.0 and $MgSO_4 \cdot 7H_2O$ 2.0 g./liter.)

Solutions, nitrogen source, g./liter	Yield of kojic acid G.	%	Sugar consumed, g.	Yield of kojic acid, based on sugar consumed, %	Mycelium, g.
			Sodium Nitrate		
A—0.62	0.13	1.2	2.2	7.5	0.298
B— .62	.19	1.8	2.0	12.1	.351
A—1.66	.35	3.2	3.0	14.8	.503
B—1.66	2.56	23.7	8.8	36.9	.750
A—5.00	0.35	3.2	3.2	13.9	.514
B—5.00	2.19	20.5	12.0	23.1	1.281
			Ammonium Nitrate		
A—0.28	2.49	23.0	6.3	50.0	0.180
B— .28	2.23	21.2	6.0	47.1	.222
A— .75	3.69	34.2	8.0	58.5	.223
B— .75	3.10	28.7	9.3	42.3	.312
A—2.25	4.83	44.7	10.0	61.1	.317
B—2.25	4.72	43.7	12.1	49.5	.511

acid produced. A range from 30 to 35° promoted rapid vegetative development with high yields of acid as shown in Table V. Respiration was apparently much accelerated at the high temperature.

In a range of sugar concentrations from 15 to 33%, inclusive, growth and acid production were satisfactory, the highest yield, based on the sugar originally present, occurring at a concentration of 20%. It is worth noting that the yields obtained in four different concentrations are practically the same when calculated on the basis of sugar consumed. With concentrations above 40% osmotic pressure apparently had an unfavorable effect, since the production of acid dropped off sharply, as indicated in Table VI.

TABLE V

EFFECT OF TEMPERATURE

Duration of culture, 12 days; concentration of commercial dextrose, 15%; volume, 75 cc.

Temp., °C.	Yield of kojic acid G.	%	Sugar consumed, g.	Yield of kojic acid, based on sugar consumed, %	Mycelium, g.
22	1.82	20.5	4.3	53.6	0.255
25	2.40	27.0	5.2	58.5	.260
30	4.00	45.0	8.1	62.6	.333
35	4.05	45.5	10.0	51.4	.370

Several experiments were carried out in which the depth of the culture medium was varied in solutions of the same sugar concentration. Some representative results are given in Table VII. As in most mold fermentations of this type, the ratio of the surface area of the mycelium to the

TABLE VI

EFFECT OF CONCENTRATION OF DEXTROSE

Duration of culture, 12 days; temperature, 35°; volume, 75 cc.

Commercial dextrose, %	Yield of kojic acid G.	%	Sugar consumed, g.	Yield of kojic acid, based on sugar consumed, %	Mycelium, g.
10	0.37	4.1	5.8	8.1	0.856
15	4.05	45.5	10.0	51.4	.370
20	5.20	48.2	12.8	51.6	.375
22.2	4.82	40.8	12.0	51.0	.373
33.3	4.65	26.2	11.5	51.3	.297
44.4	2.70	11.4	10.0	34.3	.221

TABLE VII

INFLUENCE OF CULTURE MEDIUM DEPTH

Temperature, 24–25°; concentration of commercial dextrose, 22%

Vol. of medium, cc.	Depth of medium, cm.	Surface area/vol.	Kojic acid formed in 7 days, g.	Duration of culture, days	Yield of kojic acid G.	%	Est. time for 40% yield, days
333.0	1.4	1.00	14.1	12	30.5	57.5	9
666.0	2.5	0.50	17.9	14	44.0	42.9	13
1000.0	3.5	.30	17.7	17	68.2	43.3	16
1333.0	5.8	.22	11.3	25	92.6	44.1	22

volume of the solution governs, to a large extent, the yields obtained, especially in shorter periods of culture. The maximum quantities of acid were most economically produced in 12-day cultures when this ratio had a value of from 0.3 to 0.5.

The rates of acid production, sugar consumption and vegetative growth on 20% solutions at 35° are given by the curves in Fig. 1. As soon as the supply of sugar is exhausted, the acid is utilized by the organism and slowly disappears with a corresponding diminution in the weight of the mycelium. Whether the decrease in mass of the mycelium is due to a slow autolysis cannot be definitely stated.

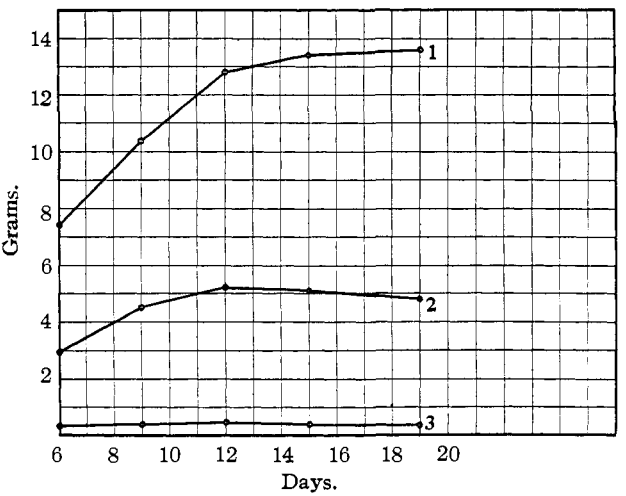


Fig. 1.—1, Sugar consumed; 2, kojic acid produced; 3, dry weight of mycelium.

The formation of this hydroxy pyrone by fungi from carbohydrates in large quantities in a short period of time is a striking phenomenon considering the relatively small quantities of related compounds found in the higher plants. Thus chelidonic acid (2,6-dicarboxy-1,4-pyrone) occurs in the leaves of lily of the valley to the extent of 2% and meconic acid (2,6-dicarboxy-3-hydroxy-1,4-pyrone) is found in opium to the extent of approximately 4%, while maltol (3-hydroxy-2-methyl-1,4-pyrone) is a minor constituent of pine needles and the bark of larches. The fact that pyrones in general react readily with ammonia to form pyridones may have an important bearing on the problem of the origin of pyridine bases found in plants, and the widespread occurrence of flavone compounds in the plant kingdom further emphasizes the importance of the pyrone nucleus in nature. Hence a detailed study of the mechanism of kojic acid formation might throw considerable light on the complete obscurity surrounding the mode of formation of these related substances in the plant cell.

Yabuta[2] thought that the formation of the acid by *A. oryzae* was connected in some manner with the oxidation changes brought about in the reduction of hexoses to the corresponding alcohols, but Kinoshita[5] pointed out that this was not necessarily so, since the organism formed kojic acid when mannitol was used as the sole carbon source. Owing to its close structural relationship to dextrose, Haworth has suggested that kojic acid resulted from the oxidation and dehydration of that hexose,[11] but the yield of acid obtained by Challenger, Klein and Walker from xylose and arabinose, as well as the fact that it is formed when levulose is used as the carbon source, casts doubt on such a mechanism. Moreover, kojic acid has been reported as being produced by Aspergilli from the following additional compounds: starch, inulin, sorbitol, dulcitol, sucrose, mannose, galactose, glycerol, glycero-β-phosphate and gluconic acid. Corbellini and Gregorini[9] believe that the pyrone nucleus is synthesized from molecules containing three carbon atoms by a reaction analogous to that brought about by the enzyme carboligase of yeast. They suggest that glyceraldehyde condenses with a molecule of another oxidation product of glycerol to give a product which by dehydration goes over to kojic acid.

The ready formation of pyrone compounds through the dehydration of appropriate acyclic polyketones is well known. Thus dimethylpyrone is formed from diacetylacetone through simple and even spontaneous loss of water at ordinary temperatures and likewise the dehydration of acetonedioxalic acid gives rise to chelidonic acid. If an analogous reaction were postulated as the final step in the formation of kojic acid, the immediate precursor of this hydroxypyrone would be 1-hydroxyacetyl-3-formyl-3-hydroxyacetone ($CH_2OHCOCH_2COCHOHCHO$). Whether this compound could result from reactive oxidation products of either methylglyoxal, glyceraldehyde or acetaldehyde is not known. The results obtained up to the present point to a synthesis of kojic acid from some reactive substance, as yet not isolated, containing two or three carbon atoms, and evidence is slowly accumulating that definitely indicates the production by fungi of such compounds of low molecular weight. The formation of acetaldehyde in the metabolism of several molds has been demonstrated,[12] but its biological significance is not yet clear. As a result of a study of this aldehyde in the citric acid fermentation induced by *A. niger*, Bernhauer concluded that there was no relation between it and the formation of the acid, as the quantity of acetaldehyde produced was entirely too small.[13] Nevertheless, the biological formation of citric acid from widely different carbon sources seems to indicate a distinct synthetic reaction. Yuill has

[11] W. Haworth, "Constitution of Sugars," London, **1929**, p. 38.

[12] C. Cohen, *Biochem. Z.*, **112**, 139 (1920); C. Neuberg and C. Cohen, *ibid.*, **122**, 204 (1921).

[13] K. Bernhauer, *ibid.*, **202**, 169 (1928).

reported the formation of appreciable quantities of ethanol but no kojic acid by a strain of *A. flavus* cultured on sucrose solution in the usual manner,[14] and the recent work of Butkewitsch and Fedoroff has established the importance of ethanol and acetic acid as intermediate substances in the reactions leading to the formation of succinic and fumaric acids by *Mucor stolonifer*.[15] From the fact that in his experiments identical fats were produced from dextrose and xylose by a strain of *Penicillium*, Barber concluded that these sugars are broken down by the organism into smaller molecules from which the fats are then synthesized.[16] It is worth noting, moreover, that up to the present no organism has been found which produces citric or kojic acids from four or seven carbon sugars or sugar alcohols. It is entirely possible that there is a single reactive substance formed by these organisms through the degradation of sugars and other carbon sources which is the starting point for the synthetic reactions brought about through their agency. Whether this substance is acetaldehyde, which serves so well in accounting for the products of yeast and bacterial fermentations, or a closely related compound, remains to be established.

Summary

The effects of variation in nutrient nitrogen, temperature, concentration of sugar and depth of culture solution on the production of kojic acid by *A. flavus* have been investigated. With 20% dextrose solutions, this organism, under favorable conditions, was found to be capable of transforming more than 45% of the dextrose present and 55% of that consumed into kojic acid in twelve days from the time of inoculation from spores.

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[14] J. Yuill, *Biochem. J.*, **22**, 1504 (1928).

[15] W. Butkewitsch and M. Fedoroff, *Biochem. Z.*, **219**, 87, 103 (1930).

[16] H. Barber, *Biochem. J.*, **23**, 1158 (1929).